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<p>(54) Title: PRODUCTION OF RECOMBINANT HUMAN INTERLEUKIN-1 INHIBITOR</p> <p>(57) Abstract</p> <p>A method for the production of commercial quantities of highly purified interleukin-1 inhibitor (IL-1<math>\beta</math>) from a recombinant host is disclosed. A preferred recombinant <i>E. coli</i> host for use in this method is also disclosed.</p>			

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PRODUCTION OF RECOMBINANT HUMAN INTERLEUKIN-1 INHIBITOR  
BACKGROUND OF THE INVENTION

In the recombinant-DNA field, many proteins have been prepared in the laboratory in an amount suitable for research purposes. However, even though techniques to produce research quantities of these proteins have been optimized, these laboratory production and purification processes are often inadequate to produce commercial quantities of the desired protein which is of a quality sufficient to be used as a human pharmaceutical.

In order to produce commercial quantities of a given protein of an appropriate quality, unique fermentation, isolation, and purification techniques are often required. Moreover, the combination of the techniques and the order in which they are practiced often affect the amount of the protein recovered and the purity of the final product.

As previously described in co-pending U.S. patent applications Serial Numbers 199,915, 238,713, 248,521 and 266,531, filed May 27, 1988, August 31, 1988, September 23, 1988, and November 3, 1988, respectively, a unique protein named human interleukin-1 inhibitor has been isolated. These applications, specifically incorporated herein by reference, also describe methods for producing recombinant human interleukin-1 inhibitor, hereinafter referred to as "IL-li," in laboratory quantities in transformed organisms useful in laboratory methods. However, these methods did not result in production of commercial quantities of IL-li of a quality suitable for administration to humans.

The present inventors have found certain combinations of fermentations, isolation, and purification techniques which are capable of producing commercial quantities of highly purified IL-1<sub>i</sub>. These methods are described in this application. As used, herein, the term "commercial quantities" is intended to mean at least several to tens to hundreds of grams of highly purified product obtained from each 100 liters of fermentation broth. By "highly purified product" is meant a material of sufficient purity to be administered to humans. In a preferred embodiment, "highly purified product" has less than 5 E.U. per dose of endotoxin and less than 0.0025% contamination by *E. coli* protein.

#### SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for the production of commercial quantities of recombinant human interleukin-1 inhibitor. This object is achieved by the methods described herein.

In order to achieve these objects, an improved strain for the production of IL-1<sub>i</sub> is described herein. That strain, named SGE90, is capable of producing at least 50 grams of highly purified IL-1<sub>i</sub> per 100 liters of fermentation broth when used in the methods described herein.

One method which is preferred for production of commercial quantities of the highly purified IL-1<sub>i</sub> described herein includes the following steps:

- (1) fermentation;
- (2) cell processing, including:

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- (a) cell recovery,
- (b) lysis, and
- (c) clarification of the lysates;

- (3) a first ion exchange step;
- (4) a second ion exchange step;
- (5) the final processing steps including concentration and diafiltration.

A third ion exchange step may be optionally added to achieve even greater product purity.

In a preferred embodiment, the fermentation step is carried out in microorganisms, particularly E. coli, while the first ionic exchange step is conducted with a column filled with the cation exchange resin S-Sepharose. Also in the preferred embodiment, the second ion exchange step is conducted with a column filled with an anion exchange resin, preferably Q-Sepharose. If the optional third ion exchange step is added, a column filled with a cation exchange resin, preferably S-Sepharose, is used.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrates various embodiments of the invention and, together with the description, serve to explain the principles of the invention.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the construction of pRJ1 and pRJ2 as set forth in Example 1.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the following examples, serve to explain the principles of the invention.

As noted above, the present invention relates to a process for the production of commercial quantities of IL-11. As used herein, the term "commercial quantities" is intended to mean that at least several to tens to hundreds of grams of highly purified product from each 100 liters of fermentation broth are produced.

As noted previously, one of the preferred methods for production of commercial quantities of IL-11 described herein includes the following steps:

- (1) fermentation of E. coli comprising a plasmid containing a DNA encoding IL-11;
- (2) cell processing, including:
  - (a) cell recovery,
  - (b) lysis, and
  - (c) clarification of the lysates;
- (3) a first ion exchange step;
- (4) a second ion exchange step;
- (5) the final processing steps including concentration

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and diafiltration.

An optional third ion exchange step may also be conducted. Such an optional step would be performed immediately after the second ion exchange step.

The fermentation and cell processing steps for use in E. coli contemplated in this invention include those routinely known to one of ordinary skill in the art. Preferred embodiments of these steps are set forth in the examples which follow. However, any comparable procedures may be inserted in the place of those preferred procedures set forth below.

The process for the production of commercial quantities of IL-li utilizes a first ion exchange column. As noted previously, the preferred column (described in Example 2), is filled with cationic S-sepharose resin. Other interchangeable resins may also be used, including but not limited to resins such as SP-C25 Sephadex, CM Sephadex, CM Sepharose, or CM cellulose. A second ion exchange column is then used for further purification of the IL-li. As noted above, in the preferred embodiment Q-Sepharose is used as an anion exchange resin in this column. In addition, other comparable resins including but not limited to resins such as DEAE-Sepharose, Q-Sephadex, or DEAE-cellulose may be employed.

In one embodiment of the present invention, a third ion exchange step is included immediately after the second ion exchange step. In this optional third step, a cation exchange column is used. This column preferably contains S-Sepharose resin, however other interchangeable resins may also be used.

Such other resins include, but are not limited to, SP-25 Sephadex, CM Sephadex, CM Sepharose, CM cellulose or CM Toyopearl.

Following these steps, the final process steps are undertaken. These include a concentration step, if desired, and diafiltration of the IL-1i. The parameters of these steps are routinely known to those of ordinary skill in the art, in light of the teachings found in the examples which follow.

Important to the operation of this process is a suitable set of quantitative analytical tools to evaluate yield and purity.

As described in greater detail in the procedures in Example 4, the assays which have been developed for these purposes include a reverse phase HPLC (RP-HPLC) assay, an ion exchange HPLC (IE-HPLC), an SDS-PAGE assay, a size exclusion assay, a trypsin peptide map, and an assay for biological activity. When tested in the first four of these assays, the highly-purified IL-1i produced by the present methods is greater than 90% pure. Preferably, when tested in the IE-HPLC, the highly purified IL-1i is at least 98% pure for both Mono Q and Mono S columns. Preferably, when tested in the SDS-PAGE assay, the highly purified IL-1i is at least 99.5% pure, and is at least 98% pure when tested in size exclusion assay. The trypsin peptide map of the highly purified IL-1i matches the pattern theoretically expected. The highly purified IL-1i demonstrates inhibition of IL-1 in the bioassay.

The following examples are provided to illustrate certain preferred embodiment of the present invention. These examples are intended to be illustrative only and are not intended to limit the scope of the claims appended hereto. All references provided in

these examples are specifically incorporated herein by reference.

EXAMPLE 1

1. Transfer of the IL-1i cDNA from a lambda phage to a Bluescript plasmid cloning vector.

The lambda phage GT10-IL-1i-2a (ATCC #40488) was digested with EcoRI and the 1.7 kb fragment carrying the IL-1i cDNA was purified by gel electrophoresis. This fragment was ligated to EcoRI-digested Bluescript SKM13-(Stratagene), resulting in the plasmid BS-IL-1i#2.

2. Development of an IL-1i expression vector using the "T7" system

A. Description of pT5T.

The T7 expression vector used for IL-1i production is called pT5T. It is essentially the same as pJU1003 [Squires, et al., J. Biol. Chem. (1988) 31:16297-16302], except that there is a short stretch of DNA between the unique Bgl2 site 5' to the T7 promoter and the Cla1 site in the tetracycline resistance gene. The sequence of this DNA is:

ATCGATGATA AGCTGTAAA CATGAGAATT GAGCTCCCCG GAGATCCTTA GCGAAAGCTA  
Cla1  
AGGATTTTTT TTAGATCT  
Bgl2

The vector was linearized with BamH1 and Sma1 restriction enzymes. The plasmid BS-IL-1i#2 was digested with PflM1 and Sca1 and the 453 bp fragment carrying the sequence coding for amino acids 4 to 152 of the mature IL-1i gene along with the termination codon and 3 bp of the 3' untranslated region was purified by polyacrylamide gel electrophoresis. Oligonucleotides with the sequences:

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5' GATCCATTGGAGGATGATTAAATGCCGCCCT 3'  
3' GTAACCTCCTACTAATTACGGC 5'

were synthesized, phosphorylated at their 5' ends and annealed. These oligonucleotides contain sequences essential for the translational coupling of the T7  $\phi$ 10 gene to the IL-1i gene. A mixture containing the annealed oligonucleotides, the linearized vector fragment and the 453 bp IL-1i gene fragment was treated with T4 DNA ligase and then used to transform the E. coli strain JM109 (See Figure 1).

B. Mutagenesis of IL-1i.

Once a plasmid was isolated and shown to have the correct sequence, it was designated pRJ1. pRJ1 carries sequences coding for a variant of the IL-1i protein. The amino-terminal sequence of this variant is Met-Pro-Pro-Ser-... rather than Arg-Pro-Ser-... which is the aminoterminal sequence of the natural human protein. The aim here was to express a protein that is as close as possible to the natural protein, and that was done by mutagenizing the DNA coding for the IL-1i protein such that it codes for Met-Arg-Pro-Ser-..., as follows. The gene for IL-1i in pRJ1 was removed by digesting the plasmid at the unique BamH1 and Pst1 sites. The 1375 bp fragment was cloned between the BamH1 and Pst1 sites of M13 mp 19 and designated M13-IL-1i1. Oligonucleotide site directed mutagenesis was performed on isolated single stand DNA of M13IL-1i1 according to the procedure described in the

BioRad Mutagene mutagenesis kit. The mutagenic oligonucleotide sequence is given below, along with the corresponding amino terminal amino acid sequence of the mutated IL-1 $\beta$ :

5' TGATTAATGCCGTCCGTCTGGGAG 3'  
M R P S G R

This mutagenesis produced M13IL-1 $\beta$ 2 which differs from M13IL-1 $\beta$ 1 in that the IL-1 $\beta$  protein encoded on this plasmid has the desired amino-terminal sequence and that the codons for Arg and Pro are those used preferably by *E. coli*.

#### C. Expression of IL-1 $\beta$ protein.

The mutagenized IL-1 $\beta$  gene was then transferred back into pT5T using the same procedure as described above. This second expression plasmid is designated pRJ2. pRJ2 was transformed into the *E. coli* strain BL21 (DE3) for expression. This strain [described in Studier and Moffat *J. Mol. Biol.* (1986) 189:113-130] contains the T7 RNA polymerase gene under control of the IPTG inducible lac promoter on a nonexcisable lysogenic lambda bacteriophage. High level expression of rIL-1 $\beta$  was achieved by growing the cells [BL21(DE3)pRJ2] in Luria broth with 15  $\mu$ g/ml tetracycline up to a cell density corresponding to an  $A_{600}$  of 0.8. IPTG was added to a final concentration of 1.0 mM and the cells were allowed to grow for four hours. The cells were harvested by centrifugation and the rIL-1 $\beta$  was purified from the lysate by standard protein chemistry technique.

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3. Development of an IL-1 $\beta$  expression vector using the "tac promoter" system.

A. Preparation of pDD1.

The plasmid pJU 1003 (Squires, et al.) was cut with Hind3 and BamH1 and fused to a synthetic Human Pancreatic Secretory Trypsin Inhibitor (HPSTI) gene whose sequence is:

ECOR1

GAATTCTGATA TCTCGTTGGA GATATTCAATG ACGTATTTG GATGATAACG  
CTTAAAGCTAT AGAGCAACCT CTATAAGTAC TGCATAAAAC CTACTATTGC

PvuI

AGGCGCAAAA AATGAAAAAG ACAGCTATCG CGATCCAGT GGCACCTGGCT  
TCCCGCTTT TTACTTTTC TGTCTGATAGC GCTACCGTCA CCGTGACCGA

GGTTTCGCTA CCGTAGCGCA GGCTGACTCT CTGGGTGCTG AAGCTAAGTG  
CCAAGCGAT GGCATCGCGT CCGACTGAGA GACCCAGCAC TTGATTAC

CTACAAACGAA CTGAACGGTT GCACTAAAT CTACAAACCCG GTATGTGGTA  
GATGTTGCTT GACTTGCCAA CGTGAATTTA GATGTTGGGC CATAACACCAT

CCGACGGTGA CACCTACCCG AACGAATGCG TGCTGTGCTT CGAAAACCGT  
GGCTGCCACT GTGGATGGGC TTGCTTACGC ACGACACGAA GCTTTGGCA

AAACGTCAGA CCTCCATCCT GATCCAGAAA TCTGGTCCGT GCTAAGTCGAC  
TTTGCAGTCT GGAGGGTAGGA CTAGGTCTTT AGACCAGGCA CGATTCAAGCTG

Hind3

CCTGCAGAAG CTT...  
GGACGTCTTC GAA...

by cutting the HPSTI gene with PvuI and Hind3 and ligating the PvuI/Hind3 fragment to the BamH1-Hind3 cut plasmid using a double stranded oligonucleotide adaptor with the sequence:

5'	GAT CCG ATC TTG GAG GAT GAT TAA ATG AAA AAG ACC GCT ATC
3'	GC TAG AAC CTC CTA CTA ATT TAC TTT TTC TGG CGA TAG
GCC AT	3'
CGG	5'

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This synthetic HPSTI gene codes for a protein consisting of the signal (or leader) peptide for the E. coli ompA protein fused to the mature HPSTI protein. Thus, the purpose of this manipulation was to incorporate sequences coding for the ompA signal peptide into pJU 1003, for work described below. The resulting plasmid is pDD1. Plasmid pDD1 was digested with BstX1 and Hind3.

B. Construction of pDD3. Addition of E. coli translational signals to the IL-1 $\beta$  cDNA.

The plasmid pT5T (described above) was cut with BamH1 and SmaI. The plasmid BS-IL-1 $\beta$ #2 was cut with the PflMI and ScaI, releasing a fragment 453 bp in length which codes for a portion of the IL-1 $\beta$  protein (see above). The BamH1/SmaI cut pT5T, the 453 bp IL-1 $\beta$  fragment, and an oligonucleotide adaptor with the sequence:

BstX1

5'	GA	TCC	ATC	GCA	GTG	GCA	CTG	GCT	GGT	TTC	GCT	ACC	GTA	GCG
3'														
	G	TAG	CGT	CAC	CGT	GAC	CGA	CCA	AAG	CGA	TGG	CAT	CGC	
	CAG	GCC	CGT	CCC	T	3'								
	GTC	CGG	GCA	G		5'								

were fused to produce the plasmid pDD2. Plasmid pDD2 was cut with BstX1 and Hind3, releasing a 499 bp fragment which codes for all of the IL-1 $\beta$  protein and a portion of the ompA signal sequence. This 499bp fragment was fused to BstX1/Hind3 cut pDD1, resulting in the plasmid pDD3.

C. Construction of pT3XI-2. Modification of pKK223-3.

The starting plasmid for this construction was plasmid pKK223-3 purchased from Pharmacia. Plasmid pKK223-3 carries a partial gene for tetracycline resistance. This non-functional gene was replaced by a complete tetracycline resistance gene carried on plasmid pBR322. Plasmid pKK223-3 was digested

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completely with SphI and partially with BamH1. A 4.4 kilobase pair fragment was gel purified and combined with a synthetic adaptor with the sequence:

5' GATCTAGAATTGTCATGTTGACAGCTTATCAT 3'  
3' ATCTTAAACAGTACAAACTGTCGAATAGTAGC 5'

and a 539 base pair fragment of DNA from a Clal - SphI digest of the tetracycline resistance gene of pBR322 (PL Biochemicals, 27-4891-01). The resulting plasmid was designated pCJ1.

Next a Xhol linker purchased from New England Biolabs was inserted into plasmid pCJ1's PvuII site to form plasmid pCJX-1. This insertion disrupts the rop gene which controls plasmid copy number. An EcoR1 fragment containing the lacI gene was purified from plasmid pMC9 [Calos, et al., Proc. Natl. Acad. Sci. USA (1983), 80:3015-3019] then inserted into the Xhol site with Xhol to EcoR1 adaptors having the sequence:

5' TCGAGTCTAGA 3'  
3' CAGATCTTAA 5'

The polylinker sequence between the EcoR1 and PstI sites in plasmid pKK223-3 was next replaced with a polylinker sequence shown here:

5' AATTCCCGGG TACCAGATCT GAGCTCACTA GTCTGCA 3'  
3' GGGCCC ATGGTCTAGA CTCGAGTGAT CAG 5'

The plasmid vector so obtained is designated pCJX1-1.

Finally, the tetracycline resistance gene was replaced with a similar gene which had the recognition sites for restriction enzymes Hind3, BamH1, and SalI destroyed by bisulfite mutagenesis. The following procedure was used to mutate the tetracycline

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resistance gene of pBR322. Plasmid pB322 was cut with Hind3, then mutagenized with sodium bisulfite [Shortle and Nathans, Proc. Natl. Acad. Sci. USA (1978) 5:2170-2174)]. The mutagenized DNA was ligated to form circular DNA, then cut with Hind3 to linearize any plasmid that escaped mutagenesis. E. coli JM109 [Yanish-Perron et al., Gene (1985) 33:103-119] was transformed with the plasmid, then plated on selective media. Plasmids were isolated from tetracycline resistance colonies and checked for loss of the Hind3 site in the tetracycline resistance gene. The successfully mutated plasmid was designated pT1. A similar procedure was followed to mutagenize the BamH1 site in pT1, yielding plasmid pT2. Plasmid pT2 in turn was mutagenized to remove the SalI site, forming plasmid pT3. A Clal-BsmH1 fragment of pT3 carrying the mutated tetracycline resistance gene was isolated and used to replace the homologous fragment of pCJXI-1 to form pT3XI-2. The mutated tetracycline resistance gene still encodes a functional protein.

D. Formation of pT3XI-2- $\Phi$ 10TC3FGFsyn. Preparing the tac promoter vector for IL-1 $\alpha$ .

Initially a "gene" for basic Fibroblast Growth Factor (FGF) was synthesized. This "gene" codes for the same sequence as that reported for FGF by Sommer et al., but uses the codons that are found preferably in highly expressed genes in E. coli. The structure of this is such that the coding portion is preceded by a translational coupler sequence (see Squires, et al., 1988) to ensure efficient initiation of translation.

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The FGF synthetic gene was first inserted into M13mp18 between the EcoR1 and Hind3 sites and sequenced. The structure of this gene is:

AATTCAGGGA TCCGATCGTG GAGGATGATT AAATGGTAC CATGGCTGCT GGCTCCATCA  
GTCCT AGGCTAGCAC CTCCTACTAA TTTACCCATG GTACCGACGA CCGAGGTAGT  
EcoRI BamHI RBS FGFstart  
 Translational Coupler 3

CTACCCGTGC GGCACTGCCG GAAGACGGTG GCTCCGGTGC TTTCCCGCCG GGCCACTTCA  
 GATGGGACGG CCGTGACGGC CTTCTGCCAC CGAGGCCACG AAAGGGCGGC CCGGTGAAGT

AAGACCCGAA ACGTCTGTAC TGTAAAACG GTGGCTTCTT CCTGCGTTAC CACCCGGATG  
 TTCTGGGCTT TGCAGACATG ACATTTTGC CACCGAAGAA GGACGCATAG GTGGGCTAC

GTCGGTGTCGA CGGCGTACGT GAAAAAAGC ACCCGCACA TCAAACTGCA GTGCAGGCTC  
 CAGCACAGCT TGCCGCCATGC ACTTTTTCC TGGGCGTGT AGTTGACGT CGACGTCCGAC

AAGAACGTG GTGTTGTATC TATCAAAGGC GTTTGCGAAA ACCGTTACCT GGCTATGAAAC  
 TTCTTGGCAC CACAACATAG ATAGTTCCG CAAACGCGTT TGGCAATGGA CCGATACTTTC

AAGACGGTC GTCTGCTGGC TAGCAAATGT GTAACTGACG AATGTTCTT CTTCGAACGT  
 TTCTGCCAG CAGACGACCG ATCGTTACA CATTGACTGC TTACAAAGAA GAAGCTTGCA

TGGAAAGCA ACAACTACAA CACCTACCGT TCTCGTAAT ACACTTCTTGC GTACGTTGCT  
 ACCTTCGT TGTTGATGTT GTGGATGGCA AGACCATTA TGTGAAGAAC CATGCAACGA

TGAAACGTA CCGGCCAGTA CAAACTGGGT TCCAAAACTG GCCCGGGTCA GAAAGCAATC  
 ACTTTGCAT GGCCGGTCAT GTTTGACCCCA AGGTTTTGAC CGGGCCCAGT CTTTCGTTAG

TGTTCCGTGC CGATGAGGCG TAAATCTTAA ACTAGTA  
 ACAAGGACG GCTACTCGCG ATTTAGAATT TGATCATTCGA  
FGFstop HinDIII

Relevant features of the gene are highlighted.

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It was then isolated by digestion with BamH1 and Hind3 and inserted into BamH1/Hind3 cut pJU1003 (Squires, et al., 1988) yielding pJU1003-synFGF. This plasmid was cut with Xba1 and Hind3 and the Xba1/Hind3 fragment carrying the FGF gene was isolated. This fragment was ligated into pT3X1-2 cut with EcoR1 and Hind3, using an EcoR1-Xba1 linker:

5' PAAT TCC ACA ACG GTT TCC CT 3'  
3' GG TGT TGC CAA AGG GAG ATCp 5'

The new plasmid is designated pT3X1-2-Φ10TC3FGFsyn.

E. Formation of pDD4. Inserting IL-1i into a tac promoter vector.

pT3X1-2-Φ10TC3FGFsyn was cut with BamH1 and Hind3, which resulted in the linearization of the 7.4 kb expression vector and the release of the insert DNA. The DNA was then cut with Ncol and Sma1, which further fragmented the insert DNA. pDD3 was digested with BamH1 and Hind3 and the 546 bp IL-1i fragment was gel purified and fused with the BamH1/Hind3-cut pT3X1-2-Φ10TC3FGFsyn 7.4 kb vector DNA fragment, resulting in the plasmid pDD4.

F. Formation of pDD5. Use of *E. coli* preferred codons.

The plasmid pDD4 carries DNA coding for the ompA signal sequence and the full length of the IL-1i protein as it was derived from the original cDNA. Plasmid pDD4 was cut with BamH1 and Spe1, thus releasing a small fragment (170 bp) carrying the sequences for the ompA signal peptide and the codons for the first 29 amino acid residues of the IL-1i protein, and the large (7.8 kb) vector fragment. The large BamH1/Spe1 vector fragment was fused to two small fragments of DNA assembled from four synthetic oligonucleotides. The sequences of these fragments are:

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5' GAT CCG ATC TTG GAG GAT GAT TAA ATG CGT CCG AGC GGC CGC  
3' GC TAG AAC CTC CTA CTA ATT TAC GCA GGC TCG CCG GCG

SacI  
AAG AGC TCC AAA AT 3'  
TTC TCG AGG TTT TAC GTC CG 5'

5' G CAG GCT TTC CGT ATC TGG GAC GTT AAC CAG AAA ACC TTC TAC  
3' A AAG GCA TAG ACC CTG CAA TTG GTC TTT TGG AAG ATG  
CTG CGC AAC AAC CAA 3'  
GAC GCG TTG TTG GTT GAT C 5'

These fragments carry sequences coding for the first 29 residues of the IL-1 $\beta$  protein using *E. coli* preferred codons [according to deBoer and Kastelein in From Gene to Protein: Steps Dictating the Maximal Level for Gene Expression (1986) Davis and Reznikoff, eds. pp. 225-283, Butterworths, NY] and a unique SacI site after the sixth codon of IL-1 $\beta$ . The resulting plasmid is called pDD5.

G. Formation of pDD6. Changes to remove secondary structure in mRNA.

Plasmid pDD5 was digested with BamH1 and SacI. The large (7.8 kb) Vector fragment resulting from this digestion was ligated to a synthetic fragment of DNA:

5' GAT CCG ATC TTG GAG GAT GAT TAA ATG CGA CCG TCC GGC CGT  
3' GC TAG AAC CTC CTA CTA ATT TAC GCT GGC AGG CCG GCA  
AAG AGC T 3'  
TTC 5'

that codes for the first 6 residues of the IL-1 $\beta$  protein, but utilizes codons that prevent the formation of any hairpin loops near the 5' end of the mRNA, especially involving the "Shine-Dalgarno" sequence or the initiation codon for the IL-1 $\beta$

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protein. This resulted in the formation of pDD6 which is the expression vector for production of IL-1 $\beta$ . Plasmid pDD6 was transformed into JM107 to yield the production strain SGE90.

EXAMPLE 2

1. Production of IL-1 $\beta$  from E. coli SGE90 Seed Growth.

Ampules of a culture of SGE90 are prepared to be used for seed as follows. A culture streak is grown on Luria agar supplemented with 15 mg/l tetracycline HCl at 37°C. A single colony is picked and grown in Luria broth supplemented with 15 mg/l tetracycline HCl at 37°C. Growth is monitored by absorbance at 660 nm (henceforth referred to as OD). When the culture reaches about 1 OD it is aseptically centrifuged and resuspended in 20% glycerol:Luria broth (1:1). It is then distributed into ampules (1.5 ml per ampule) and stored at -70°C. Working stocks are made from this cell bank by growing one ampule in Luria broth supplemented with 15 mg/l tetracycline HCl to about 1 OD, then preparing ampules as above.

The fermentor used is prepared by thawing ampules in 40°C tap water and inoculating 1 ml from the ampule prep into each of two 2 liter flasks containing 0.5 liter of Seed Media (Formula 1). The flasks are incubated for about 8 hours on a shaker at 37°C at 350 rpm. The seed OD reaches about 3-4 by this time.

500 ml of the seed culture is used to inoculate 10 liters of Fermentation Media (Formula 2). This seed tank is then grown at 37°C for 5-6 hours with pH control at 7.0, until the OD reaches approximately 5. The seed tank is then used to inoculate the fermentor.

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## 2. Fermentation.

The fermentation is carried out in 1600 liters of Fermentation Media (Formula 2). Temperature is controlled at 37°C. Dissolved oxygen is maintained at 30% (saturation with air at 3 psig). pH is controlled at 7.0 by the addition of HCl and NaOH as required.

Growth is monitored by OD. At approximately 10 OD synthesis of IL-1 $\alpha$  is induced by the addition of Isopropyl-B-D-thiogalactoside (IPTG) to a final concentration of 150  $\mu$ M. Fermentation is continued until the culture reaches an OD of about 40. Cell yield is about 150 kg solids per 1600 liters of fermentation media.

## 3. Cell recovery and washing.

Cells are recovered using a desludging centrifuge (for example an Alfa Laval BTUX 510) and washed with 150 mM NaCl. Cells are resuspended to approximately 16% solids in 150 mM NaCl and then frozen and stored at -20°C.

## 4. Cell rupture and debris removal

Fourteen kg of resuspended cells (about 2.2 kg solids) are thawed. EDTA is added to 5 mM and the cells lysed with two passes through a high pressure homogenizer. The pH is adjusted to 5.5 using 1 M acetic acid. The lysate is diluted to 20 $\pm$ 2 liters with water and clarified by centrifugation at 14,000 x G for 20 minutes.

## 5. First Ion Exchange.

(a) Column Specifications. The column used is an Amicon G300x250 filled with 7.5 liter of S-Sepharose resin

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(Pharmacia). All solutions are pumped through the column at 500 ml/min.

(b) Column Operation. The following buffer sequence is used for each cycle on the column. Buffer formulas are given in Example 4.

<u>Solution</u>	<u>Formula Number</u>	<u>Volume</u>
Equilibration	3	20 l
Clarified Cell lysate		15-25 l
Equilibration	3	20 l
Salt Gradient Elution*	3/4	40 l
NaOH Wash	5	10 l
Acetic Acid Wash	6	5 l
Storage	7	20 l

\*Salt gradient is run from 150-400 mM NaCl.

Eluate is collected by following the absorbance at 280 nm and collecting the peak eluting during the salt gradient. Recovery is about 55 g of IL-1 $\beta$  in about 10 l of the pooled fractions from 20 l of clarified cell lysate.

#### 6. Second Ion Exchange.

(a) Diafiltration. The pooled eluate is concentrated if desired using a YM10 membrane (Amicon) and then the salt is removed by diafiltration using 4 volumes of Second ion exchange Equilibration buffer (Formula 8). A precipitate which forms at this step is removed by filtration through 3 uM and 0.22 uM filters.

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(b) Column Specifications. The column used is an Amicon G180x250, filled with 5 liters of Q-Sepharose (Pharmacia). All solutions are pumped through the columns at 350 ml/min.

(c) Column Operation. The following buffer sequence is used for each cycle on the column. Buffer formulas are give in Appendix A.

<u>Solution</u>	<u>Formula Number</u>	<u>Volume</u>
Equilibration	8	20 l
Diafiltrate		5-10 l
Equilibration	8	20 l
Salt Gradient Elution*	8/9	40 l
NaOH Wash	5	10 l
Acetic Acid Wash	6	5 l
Storage	7	20 l

\*Salt gradient is run from 0 to 100 mM NaCl.

Eluate is collected by following the absorbance at 280 nm and collecting the peak eluting during the salt gradient. Recovery is about 45 g of IL-li in about 10 l of the pooled fractions from 7 l of Diafiltrate 1.

#### 7. Final Processing.

(a) Concentration and Diafiltration. The pooled eluate from the second ion exchange column is concentrated to approximately 6 l using YM10 membrane (Amicon). The material is then diafiltered against 5 volumes of Diafiltration buffer (Formula 10). Final concentration then takes place to approximately 1-2 l, with a target concentration of 10-30 g/l.

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The precipitate which forms at this step is removed by filtration through 3  $\mu$ M and 0.22  $\mu$ M filters. The final concentrate is then filtered through a 0.22  $\mu$ M filter into sterile, pyrogen free tubes and stored at -70°C. Recovery is about 80% from the pooled fractions from the second ion exchange column.

EXAMPLE 3

1. Removal of the N-terminal Methionine  
from E. coli produced IL-1i

IL-1i produced in E. coli has a sequence identical to that of IL-1i-x from human monocytes with the exception that the N-terminus has an additional methionine residue. This residue can be removed by incubating the inhibitor with the exoprotease Aminopeptidase 1 from S. cerevisiae.

10 mg of recombinant IL-1i (from the first S-Sepharose or the Q-Sepharose step of purification) is incubated with 1 mg of Yeast Aminopeptidase 1 purified as described by Change and Smith (J. Biol. Chem. 264, 6979, (1989)), in 50 mM ammonium carbonate pH 8.0 for 6 hours. The desmethylonyl IL-1i is purified from the reaction mixture by further S-Sepharose chromatography.

If wished, this step of the production process for desmethylonyl can be avoided by expressing the IL-1i in an E. coli which contains the cDNA for yeast Aminopeptidase 1 enzyme in a suitable expression vector. This E. coli should also be unable to express the gene for Aminopeptidase P (Yoshimoto et al. J. Biochem (Tokyo) 104 93 (1988) since removal of the N-terminal methionine will otherwise lead to removal of the N-terminal arginine.

It will be apparent to those skilled in the art that various modifications and variations can be made in the processes of the

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present invention. Thus, it is intended that the present invention cover the modifications and variations of these processes provided they come within the scope of the appended claims and their equivalents.

EXAMPLE 4

A. Media and formula recipes

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Formula Number	Step	Name	Components	Conc.
1	Fermentation	Seed Medium	Yeast Extract Tryptone NaCl Antifoam Tetracycline DI Water	5 g/l 10 g/l 10 g/l 0.2 ml/l 15 mg/l Q.S.
All ingredients except tetracycline are mixed and the pH adjusted to 7.5 with sodium hydroxide. Tetracycline is filter sterilized and added separately.				
2	Fermentation	Fermentation Media	NZ Amine HD KH <sub>2</sub> PO <sub>4</sub> MgSO <sub>4</sub> ·7H <sub>2</sub> O NaSO <sub>4</sub> Sodium Citrate Glycerol Antifoam Trace minerals <sup>*</sup> Thiamine HCl Tetracycline HCl DI Water	40 g/l 2 g/l 1 g/l 6 g/l 0.3 g/l 50 g/l ca. 3 ml/l 4 ml/l 10 mg/l 15 mg/l Q.S.
All ingredients through antifoam are sterilized together. Trace minerals, thiamine and tetracycline are filter sterilized and added separately.				
<sup>*</sup> Added as needed.				
*	Fermentation	Antifoam	Macol 19 GE60 antifoam	750 ml/l 250 ml/l
**	Fermentation	Trace Minerals	FeCl <sub>3</sub> ·6H <sub>2</sub> O ZnCl <sub>2</sub> CoCl <sub>2</sub> ·6H <sub>2</sub> O Na <sub>2</sub> MoO <sub>4</sub> ·6H <sub>2</sub> O CaCl <sub>2</sub> ·2H <sub>2</sub> O CuCl <sub>2</sub> ·2H <sub>2</sub> O MnCl <sub>2</sub> ·4H <sub>2</sub> O H <sub>3</sub> BO <sub>3</sub> HCl, conc. DI Water	27 g/l 1.3 g/l 2 g/l 2 g/l 2.5 g/l 1.27 g/l 3.3 g/l 0.5 g/l 160 ml/l Q.S.

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Formula Number	Step	Name	Components	Conc.
3	First Ion Exchange	Equilibration	Sodium Acetate EDTA NaCl DI/UF Water	25 mM 1 mM 150 mM Q.S.
Adjust pH to 5.5 with 5M Acetic Acid.				
4	First Ion Exchange	Elution/High Salt	Sodium Acetate EDTA NaCl DI/UF Water	25 mM 1 mM 400 mM Q.S.
Adjust pH to 5.5 with 5M Acetic Acid.				
5	First/Second Ion Exchange	NaOH Wash	NaOH NaCl DI/UF Water	0.2 M 1.0 M Q.S.
6	First/Second Ion Exchange	Acetic Acid Wash	Acetic Acid DI/UF Water	10 mM Q.S.
7	First/Second Ion Exchange	Storage	NaCl DI/UF Water	1 M Q.S.
8	Second Ion Exchange	Equilibration	Histidine EDTA DI/UF Water	20 mM 1 mM Q.S.
Adjust pH to 6.0 using 5M HCl.				
9	Second Ion Exchange	Elution/High Salt	Histidine EDTA NaCl DI/UF Water	20 mM 1 mM 100 mM Q.S.
Adjust pH to 6.0 using 5M HCl.				
10	Diafiltration	Diafiltration	NaH <sub>2</sub> PO <sub>4</sub> EDTA DI/UF Water	10 mM 0.1 mM Q.S.
Adjust to pH 7.0 using 5M NaOH.				

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B. Reverse-Phase HPLC of IL-11 -  
Non Reducing Conditions

**REVERSE-PHASE HPLC OF IL-11 - NON REDUCING CONDITIONS**

**HPLC SYSTEM:**

Beckman 114 Solvent Delivery Module  
 Beckman 165 Variable Wavelength Detector  
 Beckman System Gold Analog Interface Module 406  
 Beckman System Gold, Personal Chromatograph Software

**COLUMN:**

Brownlee RP-300 (C8)  
 (220 mm x 4.6mm, 7 micron)

**DETECTOR SETTINGS:**

Channel A, 215 nm  
 Channel B, 280 nm  
 Range: 0 - 0.05 AUFS

**MOBILE PHASE:**

A: 0.1% TFA in Water  
 B: 0.1% TFA in Acetonitrile

**GRADIENT CONDITIONS:**

<u>Time (min)</u>	<u>Percent B</u>	<u>Duration</u>
0	0	5
5	30	30 (Start Gradient)
35	50	40
75	100	5 (End Gradient)
85	0	5
95	0	End

**FLOW RATE:**

1.0 ml/min

**SAMPLE PREPARATION:**

Dilute sample to 0.1 - 0.5 mg/ml with water.

**INJECTION VOLUME:**

100 ul

**CHEMICALS:**

<u>Chemical</u>	<u>Supplier</u>	<u>Cat. No.</u>
TFA	Sigma	T-6508
Acetonitrile, HPLC Grade	Baker	9017-03

C.

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MONO Q HPLC OF IL-1 $\beta$ HPLC SYSTEM:

Beckman System Gold  
Programmable Solvent Module 126  
Scanning Detector Module 167  
Remote Interface Module  
HP Series 1050 Autosampler  
System Gold, Personal Chromatograph Software

COLUMN:

Pharmacia Mono Q HR 5/5

DETECTOR SETTINGS:

280 nm

MOBILE PHASE:

A: 20 mM TRIS, pH 7.5

B: 20 mM TRIS, pH 7.5 + 250 mM NaCl

GRADIENT CONDITIONS:

0% to 100% B in 60 minutes

FLOW RATE:

0.5 ml/min

SAMPLE PREPARATION:

None

INJECTION AMOUNT:

25 ug

D.

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## MONO S HPLC OF IL-1i

HPLC SYSTEM:

Beckman System Gold  
Programmable Solvent Module 126  
Scanning Detector Module 167  
Remote Interface Module  
HP Series 1050 Autosampler  
System Gold, Personal Chromatograph Software

COLUMN:

Pharmacia Mono S HR 5/5

DETECTOR SETTINGS:

280 nm

MOBILE PHASE:

A: 25 mM NaAc, pH 5.5 + 1 mM EDTA

B: 25 mM NaAc, pH 5.5 + 1 mM EDTA + 500 mM NaCl

GRADIENT CONDITIONS:

0% to 60% B in 36 minutes.

FLOW RATE:

0.5 ml/min

SAMPLE PREPARATION

None

INJECTION AMOUNT:

25 ug

E.

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## SIZE EXCLUSION HPLC OF IL-1i

HPLC SYSTEM:

Beckman 114 Solvent Delivery Module  
Beckman 165 Variable Wavelength Detector  
Beckman System Gold Analog Interface Module 406  
Beckman System Gold, Personal Chromatograph Software

COLUMN:

Bio-Sil TSK 250 (7.5 mm x 30 cm)

DETECTOR SETTING:

280 nm  
Range: 0 - 0.2 AU

MOBILE PHASE:

25mM Na Acetate and 0.5M NaCl, pH 5.5

FLOW RATE:

0.5 mls/min

SAMPLE PREPARATION:

Dilute IL-1i solution with mobile phase to a final concentration of approximately 2 mg/ml

INJECTION VOLUME:

50 ul

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F. REDUCING SDS PAGE OF IL-11

GEL PREPARATION:

Follow procedure outlined by Lammli in J. Mol. Biol., 80, 575-599 (1973).

SEPARATING GEL:

Acrylamide	15%
TRIS pH 8.8	375 mM
SDS	0.1%

STACKING GEL:

Acrylamide pH 6.8	5%
SDS	0.1%

SAMPLE PREPARATION:

Sample is diluted 1:1 with Sample Buffer. The samples are then heated for 15 minutes at 65°C, spun and loaded onto the gel.

SAMPLE BUFFER:

TRIS pH 6.8	250mM
SDS	2.5%
2-Mercaptoethanol	5%
Glycerol	12.5%

ELECTROPHORESIS CONDITIONS:

50 V until samples have reached the separating gel.  
100V until the bromophenol blue runs out of the gel.

STAINING:

Ethanol	45.4%
Acetic Acid	9.0%
Water	45.5%
Coomassie Brilliant Blue	2.5g

Stain overnight at room temperature with gentle shaking.

DESTAINING:

Methanol	30.0%
Acethic Acid	12.5%
Water	57.5%

Destain overnight or until background is clear at room temperature.

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MOLECULAR WEIGHT STANDARDS:

Low Molecular Weight Range (BRL):

<u>Protein</u>	<u>Reported MW</u>
Insulin (A and B)	2,300 and 3,400
Bovine Trypsin Inhibitor	5,200
Lysozyme	14,300
$\beta$ -Lactoglobulin	18,400
Alpha-Chymotrypsin	25,700
Ovalbumin	43,000

5 ug of the protein mixture is loaded onto the SDS PAGE gel.

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G. TRYPSIN PEPTIDE MAP OF RECOMBINANT HUMAN IL-1 $\beta$

PROCEDURE:

1. Reagents

- 1.1 Trypsin sequencing grade; Boehringer Mannheim GmbH.
- 1.2 Urea ultra pure; BRL.
- 1.3 Milli-Q water.
- 1.4 Trifluoroacetic acid; Pierce.
- 1.5 HPLC grade acetonitrile; J.T. Baker.
- 1.6 Tris
- 1.7 CaCl<sub>2</sub>

2. Equipment

2.1 HPLC system

Beckman 114 Solvent Delivery Module  
Beckman 165 Variable Wavelength Detector  
Beckman System Gold Analog Interface Module 406  
Beckman System Gold, Personal Chromatograph  
Software

2.2 Column

BrowLee RP-300 (C8)  
(220mm x 4.6mm, 7 micron)

2.3 Heating/cooling water bath

3. Solution

- 3.1 Trypsin; Dissolve 0.1 mg in 0.1 ml of 0.1M HCl;  
Store frozen at -20°C, stable for months without  
loss of activity.
- 3.2 Urea: 8 M urea in Milli Q water, make fresh  
daily.
- 3.3 2M Tris HCl pH 8.0 and 0.1M Tris HCl pH 8.0.
- 3.4 3mM CaCl<sub>2</sub>

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#### 4. Method

- 4.1 Denature of IL-1 $\beta$  in 6M urea and 0.1M Tris pH 8.0 final concentration at about 3 mg/ml protein for 10 minutes at 37°C.
- 4.2 Dilute into a solution of 0.1M Tris HCl pH 8.0 containing 0.3mM CaCl<sub>2</sub> (1:2 vol/vol) to give a final concentration of 2M urea.
- 4.3 Add trypsin solution (solution number 3.1) to give 1% by weight of the protein. Mix well.
- 4.4 Incubate at 37°C for 1 hr and additional 1% by weight of trypsin is added.
- 4.5 Stop digest after an additional 3 hrs by freezing at -20°C or by acidification with 10% trifluoroacetic acid, final concentration 0.1%.
- 4.6 Inject onto the HPLC column.

#### 5. Reverse-phase of peptide fragments produced by trypsin digestion.

- 5.1 HPLC system and column as in Section 2.

##### 5.2 Detector settings:

Channel A: 215 nm  
Channel B: 200 nm  
Range: 0-0.5 AU

##### 5.3 Mobile phase:

A: 0.1% TFA in water  
B: 0.1% TFA in acetonitrile

##### 5.4 Gradient conditions

<u>Time (min)</u>	<u>Percent B</u>	<u>Duration</u>
0	0	0
5	40	80
85	100	5
95	0	5
120	0	End

##### 5.5 Flow rate

1.0 ml/min

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5.6 Sample preparation

none

5.7 Injection volume

50 to 100 ul

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#### H. IL-1i Bioassay.

The assay for IL-1 inhibitor is based on an IL-1 assay developed by S. Nakai, K. Mizuno, M. Kaneta and Y. Hirai. (Biochem. Biophys. Res. Comm. 154:1189-1196. 1988). The principle of this assay is that prolonged exposure to IL-1 is cytotoxic to the human melanoma cell line A375. The cytotoxicity is mediated via the IL-1 receptor. IL-1i antagonizes this cytotoxicity in a dose dependent manner by competing with IL-1 for binding to the IL-1 receptor. The level of toxicity can be quantitated by staining the live cells with crystal violet, extracting the stain from the cells by overnight incubation in 100% ethanol, and measuring the optical density of the extracted stain with a spectrophotometer. The rationale for the use of the A375 melanoma cell bioassay is that it is a simple and direct method for measuring both IL-1 and IL-1i activity. Most other assays that have been described in the literature depend on the ability of IL-1 to activate other products, such as prostaglandin E2 and lactic acid in fibroblasts, and interleukin-2 in T-cells. These secondary products are then measured in order to determine the level of IL-1 present. Although all of these IL-1 activities are receptor mediated, the existence of more than one stage makes these alternative assays cumbersome and subject to a greater probability of error.

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WHAT IS CLAIMED IS:

1. A method for the production of commercial quantities highly purified interleukin-1 inhibitor (IL-1 $\beta$ ) comprising:
  - (1) fermentation of E. coli comprising a plasmid containing a DNA encoding IL-1 $\beta$ ;
  - (2) cell processing, including:
    - (a) cell recovery,
    - (b) lysis, and
    - (c) clarification of the lysates;
  - (3) a first ion exchange step;
  - (4) a second ion exchange step;
  - (5) the final processing steps including concentration and diafiltration.
2. The method of claim 1 wherein the plasmid is pDD6.
3. The method of claim 1 wherein the first ion exchange step uses a column packed with a cationic resin.
4. The method of claim 3 wherein the cationic resin is selected from the group consisting of S-Sepharose, SP-C25 Sephadex, CM Sephadex, CM Sepharose, CM cellulose, or CM Toyopearl.
5. The method of claim 4 wherein the cationic resin is S-Sepharose.
6. The method of claim 1 wherein the second ion exchange step uses a column packed with an anionic resin.
7. The method of claim 6 wherein the anionic resin is selected from the group consisting of Q-Sepharose, DEAE-Sepharose Q-Sephadex and DEAE cellulose.

8. The method of claim 7 wherein the anionic resin is Q-Sepharose.

9. The method of claim 1 which further comprises a third exchange ion exchange step conducted immediately prior to the final processing steps.

10. The method of claim 9 wherein the third ion exchange step is conducted using a cationic resin.

11. The method of claim 10 wherein the cationic resin is selected from the group consisting of S-Sepharose, SP-C25 Sephadex, CM Sephadex, CM Sepharose, CM cellulose, or CM Toyopearl.

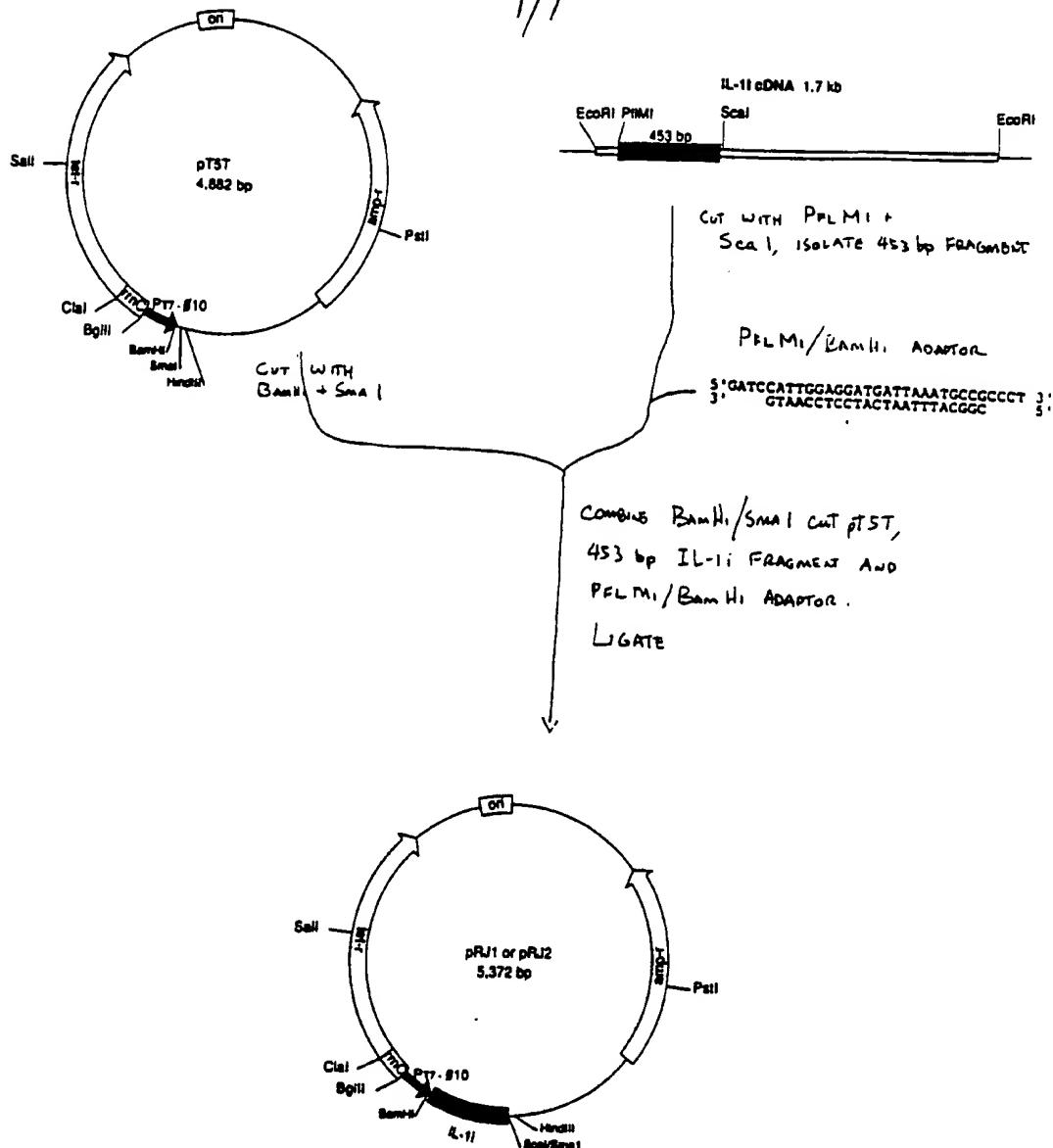
12. The method of claim 11 wherein the cationic resin is S-Sepharose.

13. A transformed E. coli host comprising at least one plasmid containing a DNA sequence encoding IL-1i which is capable of producing commercial quantities of highly purified IL-1i.

14. The plasmid pDD6.

15. Interleukin-1 inhibitor bound to an anionic resin.

16. Interleukin-1 inhibitor bound to a cationic resin.



**IL-11 Insert**  
 D P L E D D I H R P IL-11 coding sequence  
 GATTCATGGAGGATTTAAATGCCCGCT-----AGCTGG  
 BamHI  
 BamHI

**IL-12 Insert**  
 D P L E D D I H R P IL-11 coding sequence  
 GATTCATGGAGGATTTAAATGCCCGCT-----AGCTGG  
 BamHI  
 BamHI

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06979

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) :  
 According to International Patent Classification (IPC) or to both National Classification and IPC  
 IPC(5): C12N 1/21; C07K 3/00  
 U.S.CI.: 435/252.33; 530/350

**II. FIELDS SEARCHED**

Minimum Documentation Searched <sup>4</sup>	
Classification System	Classification Symbols
U.S.	— 435/320, 252.33; 530/350, 416

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched<sup>5</sup>

Chemical Abstracts Data Base (CAS) 1967-1991.

Key words: interleukin inhibitor, protein purification, anion, cation exchange

**III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>14</sup>**

Category <sup>6</sup>	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>16</sup>	Relevant to Claim No. <sup>17</sup>
	See Attachment	

\* Special categories of cited documents: <sup>18</sup>  
 "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
 "&" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search:

22 February 1991

International Searching Authority:

ISA/US

Date of Mailing of this International Search Report:

**09 APR 1991**

Signature of Authorized Officer:

*Joan Ellis*

(1) Attachment to form PCT/ISA/210  
Continuation in part III  
Documents to be Considered Relevant

- Y WO, A, 89/01946 (DAYER ET AL) 09 March 1989, 1-12,  
see pages 17-19 and 27. 15, 16
- Y Journal of Experimental Medicine, Volume 168, 1-12,  
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"A Human Urine-Derived Interleukin<sup>1</sup> Inhibitor.  
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1778, especially pages 1768-1770.
- Y Nature, Volume 343, No. 6256, issued 25 January 1990, 1-12,  
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G. Sofer, "Chromatographic Removal of Pyrogens", 15, 16  
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- Y Pharmacia Fine Chemicals Catalogue, issued 1980, 1-12,  
"Ion Exchange Chromatography", pages 29-31, see 15, 16  
entire document.
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Chromatographic Purification Scheme for  
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Figure 3.